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Exhibit 13

GENERATION OF TRANSGENIC DAIRY CATTLE USING *'IN VITRO'* EMBRYO PRODUCTION

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We have combined gene transfer, by microinjection, with *'in vitro'* embryo production technology, enabling us to carry out non-surgical transfer, to recipient cows, of microinjected embryos that have been cultured from immature oocytes. Using this approach, we have established 21 pregnancies from which 19 calves were born. Southern blot analysis proved that in two cases the microinjected DNA had been integrated in the host genome.

Heterologous protein production in the mammary gland of dairy animals may become an important alternative to cell-culture based expression systems. The feasibility of this technology in the mouse system has been demonstrated in several reports in which high level, tissue-specific expression of foreign proteins in milk was obtained[1–5]. Several groups have reported limited success of germline transformation by applying essentially the same approach to other species, in particular animals with a relatively short generation time such as rabbits, sheep and pigs[6–11]. In these experiments, the supply of fertilized oocytes was, like that in the murine system, based on superovulation followed by *'in vivo'* fertilization. After surgical removal of the zygotes followed by microinjection, embryos were allowed to develop in the oviduct of recipients into which they were placed via another surgical procedure.

Dairy cows would seem to be the optimal species for production of very large quantities of heterologous protein in the mammary gland since they can produce over 10,000 liters of milk per year that contains 35 grams of protein per liter[12]. However, the generation interval of cattle is about 2 years and cows normally produce only one offspring per gestation. In addition, the logistics of supplying the large numbers of bovine zygotes that are required for the production of transgenic animals from live animals using conventional procedures is cumbersome. Moreover, superovulation and artificial insemination followed by flushing of oviducts of donor cows and oviductal transfer to recipients is very costly because of the two surgery steps involved.

Here we report the generation of transgenic dairy cattle based on a novel approach in which gene transfer is combined with an *'in vitro'* embryo production procedure, thus enabling non-surgical transfer of microinjected embryos that have developed from immature oocytes. Using this approach we demonstrate, for 2 cases, the successful incorporation of microinjected DNA in the genome. The integrated DNA construction contains signals for directing expression of the human iron binding protein, lactoferrin, to the mammary gland.

RESULTS

Oocyte maturation and fertilization. Bovine oocytes were collected by aspiration of follicles present on ovaries obtained from local slaughterhouses. For this study a total of about 2500 oocytes were used. On average we performed two aspiration sessions per week. The yield of aspirated oocytes was highly variable from day to day, with a mean daily number of about 150. Maturation and fertilization were analyzed by cytological analysis. Maturation was defined as the breakdown of the nuclear membrane, the appearance of the first polar body and a metaphase plate. For fertilization, frozen semen from three different bulls was used, each with excellent characteristics with respect to genetic background, field perfor-

TABLE 1 Efficiencies of the steps involved in the process from immature bovine oocytes to transgenic calves.

Step	Total No.	Percent*
oocytes	2470	—
matured	2297	93
fertilized	1358	61
injected	1154	85
survival	981	85
cleavage	687	70
transferred	129‡	19
pregnant	21	21
integration	2	10

*Percentages indicate the proportion of embryos or cells that successfully complete each step.
‡Sixty-nine transfers of single blastocytes resulting in 7 pregnancies; 30 transfers of twinned embryos, resulting in 14 pregnancies.

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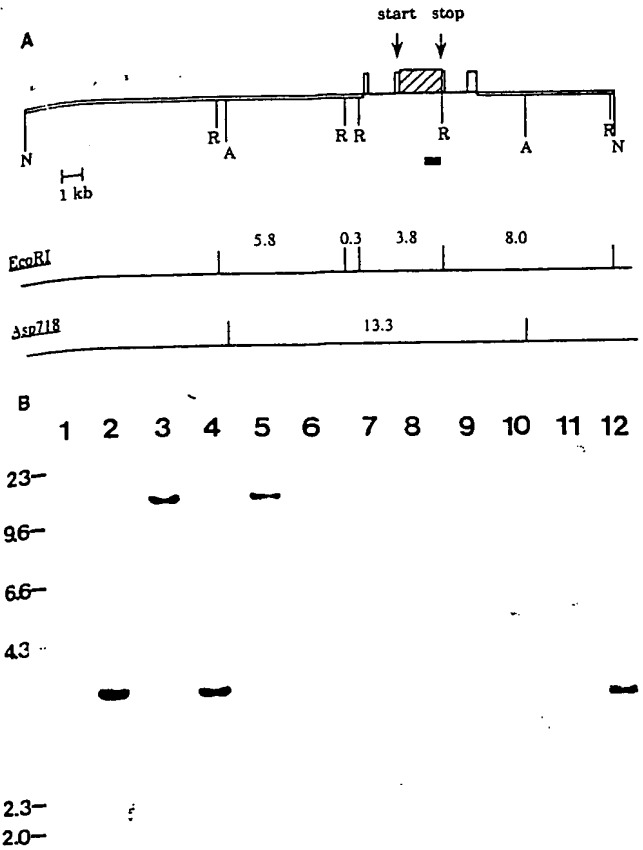

FIGURE 1 (A) Structure of the bovine casein-hLF transgene. The coding sequence of the hLF cDNA is depicted by a hatched box. The position of the translational start and stop codon is indicated. The 5′ and 3′ untranslated regions are encoded by αS1-casein exons (open boxes). Intervening sequences interrupting these exons are represented by a single line. The expression unit is surrounded by flanking sequences derived from the bovine αS1-casein gene (indicated by a double line). Positions of restriction enzyme sites are indicated by the following symbols: R, EcoRI; A, Asp718; N, NotI; The NotI sites are not present at the indicated positions in the bovine αS1-casein gene itself, but were introduced by synthetic linkers. The black bar represents the position of the probe used to detect the presence of the transgene. Sizes of the fragments (in kbp) obtained after digestion with EcoRI or Asp718 are shown at the bottom. (B) Southern blot analysis of DNA extracted from various tissues. Ten μg of DNA was loaded per lane. Fragment size markers in kbp. (HindIII digest of lamda DNA) are indicated on the left. Lane 1, EcoRI digested human DNA (isolated from blood), lane 2, Eco RI digested DNA from calf #4 isolated from blood; lane 3, Asp718 digested DNA from calf #4 isolated from blood; lane 4, EcoRI digested placental DNA from calf #4; lane 5, Asp718 digested placental DNA from calf #4; lane 6, EcoRI digested DNA from calf #15 isolated from blood; lane 7, Asp718 digested DNA from calf #15 isolated from blood; lane 8, EcoRI digested DNA from calf #15 isolated from ear tissue; lane 9, Asp718 digested DNA from calf #15 isolated from ear tissue; lane 10, EcoRI digested placental DNA from calf #15; lane 11, Asp718 digested placental DNA from calf #15; lane 12, EcoRI digested DNA isolated from the tail of a transgenic mouse harboring the same construct.

mance and ease of calving. For each batch of semen the '*in vitro*' fertilization conditions (heparin concentration and sperm number) were optimized to obtain normal fertilization rates ranging from 50 to 70% as determined by the presence of two pronuclei and a sperm tail. We used either one of two techniques for selection of motile sperm: the swim-up technique[13] and centrifugation through a Percoll gradient (J. Parrish, personal communication). No significant differences in fertilization rates between these methods were recorded. The efficiencies of these and the following steps are shown in Table 1.

Pronuclear injection. In order to visualize pronuclei, fertilized oocytes were centrifuged at 14,500× g for 8 minutes[14]. The time window in which pronuclei could be visualized appeared to be smaller than the period in which murine pronuclei are visible. Cytologically, i.e. after fixation and staining with aceto-orcein, pronuclei are clearly detectable from 16 hours up to 23 hours after fertilization, whereas using interference contrast optics pronuclei can be visualized after centrifugation between 18 hours and 23 hours after fertilization. The number of oocytes in which pronuclei could be visualized during this period was about 10% smaller than the number that was expected based on the cytological data. Possibly, some nuclei are hidden behind the yolk cap that is formed after centrifugation. Pronuclear injection was performed essentially as described[15]. About 10% of the zygotes collapsed after microinjection and were discarded.

Embryo development. After microinjection, the embryos were transferred to microdrops of medium conditioned by bovine oviductal epithelial cells as described[16]. Embryo development was evaluated 9 days after the start of maturation. The development rate varied by about 5% depending on the sperm donor used. In addition, microinjected embryos developed, in general, slower than control embryos that had been centrifuged but not microinjected. The formation of the blastocyst cavity was delayed by approximately 1 day. Also, fewer microinjected embryos developed to the morula/blastocyst stage than control embryos (19% vs. 20–25%, Table 1).

Embryo transfer. The synchronization schedule was set up so that recipients started estrous on the same day at which oocytes were aspirated from slaughterhouse ovaries (i.e. start of maturation is day 1). Recipients received 9-days old embryos, at which time they have developed to the compact morula or early blastocyst stage. These embryos are one day ahead in development compared to the stage of the estrous cycle of the recipients. In case of two microinjection sessions on subsequent days, one group of recipients was used that were in synchrony with the first batch of oocytes collected. Transfers of embryos that developed from oocytes aspirated on the day of the start of estrous gave better results than embryos from oocytes obtained one day later. Due to the somewhat delayed development of microinjected embryos, there appeared to be a better synchrony between the recipients and the first group of embryos. Recipients received two embryos when the quality grade (according to Linder and Wright[17]) was fair to poor and only one single embryo when the quality grade was excellent to good. Each pregnant recipient that received 2 embryos carried only one fetus to term. The overall pregnancy rate was 21%, which is significantly less than the rates reported by others with non-microinjected embryos which had developed '*in vivo*'[18,19]. In the experiments reported here, no transfers with non-injected embryos were performed.

A total of 21 pregnancies were established (confirmed by rectal palpation 45–60 days after transfer). During pregnancy 2 fetuses were lost. One recipient aborted spontaneously for unknown reasons at 7.5 months of gestation. The second fetus, collected at slaughter of the recipient at 3 weeks after the calculated day of parturition, was a full grown dead calf having an abnormal embryonic development called 'schistosoma reflexum'. In both cases no intact DNA could be isolated for analysis. Nineteen calves were born after normal pregnancies. One of these calves died during parturition, and a second, 24 hours after birth, because of pneumonia following accidental

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inhalation of milk. A third calf, born after a pregnancy of 10 months and with a body weight of 70kg was euthanized at an age of 3 weeks. Pathological analysis indicated that the animal was suffering from sepsis due to chronic omphalephlebitis. Tissues that could be analyzed from the three dead calves contained no integrated human lactoferrin (hLF) sequences. Therefore, the cause of their death is unlikely to be related to transgene integration. The remaining 16 calves are in excellent health.

Structure of the transgene. The fragment used for injection was designed to express hLF in the mammary gland of a lactating cow. It consists of the coding region of the hLF cDNA[20] fused to the bovine αS_1-casein signal sequence and flanked by the 5′ and 3′ untranslated regions of the bovine αS_1-casein gene. Both untranslated regions are interrupted by an intervening sequence. These introns were included since several groups have shown that the presence of intervening sequences can dramatically increase expression of cDNA-based constructs both in tissue culture and in transgenic animals[21,22].

Expression of the cDNA is controlled by regulatory elements within 15 kbp of 5′-flanking and 6 kbp of 3′-flanking sequences from the αS_1-casein gene. These sequences have been shown to contain elements that are responsible for tissue-specific expression of heterologous genes in transgenic mice[5] (and our own unpublished observations). A schematic drawing of the casein-hLF transgene is shown in Figure 1.

DNA analysis. DNA was isolated from placenta, blood and ear tissue from all calves. Southern blot analysis using hLF cDNA as a probe indicated that in tissues of two calves (#4 and #15) transgene sequences had been integrated into the host genome. Calf #15 (a female) was mosaic for integration of the transgene: placental tissue was positive, whereas in blood and ear tissue no hLF sequences could be detected. The copy number in the placenta was 1–2. The restriction enzyme map of the transgene was different from that expected based on the map of the casein-hLF plasmid (Fig. 1) and based on the pattern obtained in many individual transgenic mice (data not shown). Apparently, a rearrangement had occurred involving a deletion of part of the DNA construct. It is not clear whether this rearrangement event is related to the fact that the transgene could not be detected in all tissues. In mice it has been shown that over 30% of all transgenic animals born are mosaic[23].

Calf #4 (a male) showed, in all three tissues, the same hybridization pattern that was identical to the expected one. Restriction digestions with different enzymes indicated that head-to-tail concatemers of intact copies had integrated and there was no indication of rearrangements. Copy numbers were estimated by comparing the intensities of the transgenic band with bands resulting from hybridization of the hLF probe to human DNA (Fig. 1). In calf #4 between 5 and 10 copies of the transgene had integrated in all three tissues examined.

DISCUSSION

This work proves the technical feasibility of transgenesis in the bovine system: in 2 out of 19 calves born from microinjected zygotes, the introduced DNA was integrated into the host genome. In parallel experiments, the transgenesis rate in mice that received the same casein-hLF construct was about 10% (data not shown). Thus, based on the limited number of animals born, we tentatively conclude that the transgenesis rates in cattle and mice are, in our hands, the same.

Most attempts to produce transgenic cattle have relied partly or totally on *in vivo* procedures[24,25]. Fertilized oocytes were retrieved from superovulated and artificially inseminated cows. Microinjected zygotes were transferred by surgery either directly into the oviduct of recipient cows or into temporary hosts like sheep or rabbits. Obviously, the *'in vivo'* production of zygotes is much more labor intensive than the *'in vitro'* production. Also a larger number of additional animals and veterinary surgeons are involved. Another major disadvantage of the *'in vivo'* procedure is that the stage of pronuclear development at which the zygotes are isolated and processed varies considerably[26], and therefore the most suitable time for microinjections cannot be determined. In addition, up to 50% of the embryos transferred into ligated oviducts in living animals cannot be recovered[27]. Finally, the development of the embryos cannot be followed in the oviduct of temporary hosts, and therefore the stage at which the embryos are recovered is unpredictable. Some of the disadvantages of the *'in vivo'* procedure may be compensated to some extent by the fact that embryos produced by this method have, in general, a better developmental potential[28].

All the disadvantages described above are circumvented when using the *'in vitro'* procedures described here. A large number of aspirated oocytes are matured and fertilized simultaneously and their pronuclei can be injected within a short time. Development of the individual embryos can be monitored closely, which makes it possible to transfer embryos at very specific stages of development. The *'in vitro'* culture of embryos up to the morula/blastocyst stage also allows for additional manipulations such as biopsy of blastomers for transgenesis detection, sexing and cloning. It is expected that the incorporation of these, in part established, technologies in our *'in vitro'* program will further reduce the number of recipients needed to generate (larger numbers of) transgenic cattle.

The procedures described in this paper make use of oocytes obtained from ovaries of slaughtered cows. As a consequence, the genetic background of these animals is unknown. Recent developments in the efficient retrieval of oocytes from live animals using echoscopy[29,30] make it possible to generate transgenic calves that have a defined genetic background both from the maternal and the paternal lineage.

EXPERIMENTAL PROTOCOL

Oocyte source. Ovaries were collected at a local abattoir and transported to the laboratory in a insulated container at 30–32°C. Oocytes, together with follicular fluid, were aspirated from 2–6 mm diameter follicles and pooled into 50 ml conical tubes. Cumulus-oocyte complexes (COC) were allowed to settle into a pellet, after which the supernatant was discarded and the pellet washed in 50 ml TL-Hepes[30]. COC, containing several intact unexpanded cumulus cell layers, were selected and isolated under a dissecting microscope at 15× magnification, washed four times in 10 ml TL-Hepes, once in 2–3 ml TCM199+10% fetal calf serum (M199)[30] and then transferred to 100 µl droplets of M199 medium under paraffin oil (20 COC/droplet). COC were incubated for 23 h in a humidified atmosphere of 5% CO_2 in air at 39°C.

***'In vitro'* fertilization.** Oocytes were fertilized with frozen-thawed-sperm obtained from three different bulls in artificial insemination service. Sperm capacitation was facilitated with heparin[13]. Since sperm from individual bulls respond differently to specific fertilization conditions, semen from each lot was tested in advance to determine optimal heparin and sperm concentration required to maximize normal fertilization frequency and to minimize polyspermy. Fertilization conditions for a given bull were selected after screening at heparin concentrations of 0.0, 1.0 and 10.0mg heparin/ml, and at 1.0, 2.0 and 4.0 × 10^6 motile sperm/ml. Since the proportion of sperm that survives freezing and thawing varies from bull to bull (approximately 30–60% for the bulls used here) sperm preparations were enriched for live, motile sperm by a "swim-up" procedure[13]; alternatively, sperm were centrifuged through a percoll gradient (J. Parrish, person-

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communication). After isolation of the motile portion, sperm were counted on a hemocytometer, diluted to an appropriate concentration to yield a 25-fold concentrated stock. The fertilization medium consisted of TALP medium[31] supplemented with 5.0–10.0 mg/ml heparin (from porcine intestinal mucosa, 177 IU/mg; Sigma)[13] and if the cumulus was removed prior to fertilization, 1mM hypotaurine, 10mM penicillamine, 20mM epinephrine and 2mM sodium metabisulfite. Matured COC were selected on the basis of expanded cumulus masses for fertilization, washed once in 10 ml fertilization medium, and either added directly to fertilization droplets, or first stripped of their cumulus investment by gentle pipetting through a small-bore, fire-polished pipet and then added to the droplets. Finally, sperm cells were added to a final concentration of 1×10^6–2.0×10^6/ml. After 16–24 h, presumptive zygotes were removed from fertilization droplets. At this point, 20–30 zygotes for each experiment were fixed in 3:1 ethanol:acetic acid for 24 h, stained with 1% aceto-orcein (in 40% acetic acid), and examined to determine fertilization frequency (percentage of sample with 2 pronuclei and a sperm tail). The remaining oocytes were then prepared for microinjection.

Microinjection. The 26 kbp casein-hLF fragment used for microinjection was released by NotI digestion and purified by agarose gel electrophoresis and electroelution. The final DNA concentration was adjusted to 2.5 μg/ml. Batches of 50 cumulus-intact fertilized oocytes were stripped either as described above or by vortexing 2 minutes in 2ml Tl-hepes medium in a 10ml conical tube. In order to visualize the pronuclei, cumulus free oocytes were centrifuged in 1ml TL-hepes medium 8 minutes at 14,500× g in an Eppendorf centrifuge[14]. Microinjection was performed essentially as described[15].

Embryo culture. Embryos were cultured from the zygote to the compact morula or blastocyst stage in oviductal-tissue conditioned medium[16]. Oviducts were obtained at slaughter and transported at ambient temperature. Luminal tissue from 2–4 oviducts (1–2 cows) was harvested by gently scraping intact oviducts on the outside with a glass slide. The extruded material was washed 5 times in 10 ml TALP-Hepes and diluted in M199 to a tissue:media ratio of 1:50. Media were conditioned in 50 ml "T" flasks containing five ml of oviduct tissue suspension. Conditioned medium was prepared 48 h later from the supernatant after centrifuging tissue suspensions at 13000× g for 10 min., divided into 1.0 ml aliquots and stored at −20°C until used. Conditioned media frequently contained a proteinaceous precipitate after thawing, which was removed by centrifugation. Droplets were covered with paraffin oil and were incubated for 2 h to permit pH to equilibrate prior to adding zygotes. Zygotes were placed in culture droplets within 2 h after microinjection. Initial cleavage (>2 cells) was assessed 42 h after adding sperm. Media were not changed during the course of incubations. Criteria for normal development consisted of attainment of the compact morula or blastocyst stage.

Embryo transfer. Estrous in recipient cattle was synchronized with a 9-day Norgestamet (Intervet, Boxmeer, The Netherlands) treatment (administered in an ear implant according to the manufacturer), and a 500 μg dose of cloprostanol given on day 7 of the Norgestamet treatment. Estrous occurred within 2–3 days after implant removal. Embryos were transferred non-surgically to recipient heifers 5–7 days after estrous (1–2 embryos/uterine horn). Pregnancy was determined by rectal palpation at 45 to 60 days of gestation.

DNA analysis. DNA extraction, Southern blot analysis and hybridization were performed according to standard procedures[32]. The probe used in the Southern blotting experiment was a 758 bp EcoRV-EcoRI fragment covering the 3' part of the hLF cDNA[20].

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